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FOREWORD

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Introduction

The Role of EGF Receptor Negative Regulatory Components in Breast Cell Growth

Amplification of the epidermal growth factor receptor (EGF-R) is correlated with a poor prognosis for breast cancer patients (1). In addition, several different areas of research further support the idea that EGF-R expression levels are important in the development of breast cancer (2-7). These studies include *in vivo*, *in vitro* and transgenic animal studies (2-7).

The EGF-R is a transmembrane tyrosine kinase (8). Following binding to one of its ligands, the EGF-R is rapidly phosphorylated on C-terminal tyrosine residues. Phosphorylation allows the receptor to initiate signaling cascade via numerous interactions with other protein molecules (8-10).

The EGF-R is negatively regulated both spatially and covalently (8, 11-15). After binding ligand, the EGF-R is rapidly internalized via coated pit mediated endocytosis. The internalized receptors are then sorted through the endocytic apparatus and either recycled back to the cell surface or degraded in lysosomes. Lysosomal degradation leads to a decrease in the overall EGF-R receptor mass following ligand stimulation and is referred to as receptor down-regulation (11-15). Alternatively, phosphorylation of the EGF-R on serine or threonine residues leads to receptor desensitization (8). Desensitized receptors have a decreased affinity for ligand and reduced tyrosine kinase activity. Both receptor down-regulation and desensitization control the extent and duration of EGF-R signaling in response to ligand stimulation (8).

The MDA-MB-468 malignant breast cell line and its "S" variant lines generated by Filmus *et al* have been used to correlate EGF-R levels and tumorigenicity (4 and 5). The MDA-MB-468 cell line expresses 1.9×10^6 EGF-R per cell due to genomic amplification and is growth inhibited by exogenous EGF (4 and 16). Filmus *et al* selected the "S" variant cell lines from the parental MDA-MB-468 cell line by growth in EGF containing media. All of the variants had lost the parental cell line EGF-R genomic amplification. The S1 variant expresses 1.6×10^4 EGF-R per cell and the S11 variant expresses 6.6×10^4 receptors per cell. The parental cell line has a faster growth rate than either variant and forms larger more aggressive tumors when injected into nude (4 and 5).

These results suggested that the loss of EGF-R amplification may have directly led to the decreased tumorigenicity seen with the variants.

These observations led us to hypothesize that amplification of the EGF-R in the absence of concomitant increases in its negative regulatory components would lead to dysregulated kinase activity and uncontrolled receptor signaling. Because EGF-R kinase activity determines both its mitogenic and transforming ability, dysregulation would lead to uncontrolled growth and contribute to the formation of cancers (9). This hypothesis is currently being tested.

Statement of Work

Part I.

Determine the percent reduction of EGF-R kinase activity following PMA or EGF induced desensitization for all cell types being

Months 1-12

Part II. A

Transfect all cell types with mutant receptors and characterize the total receptor number for each transfectant.

Months 13-24

Body of Report

Several conclusions were made in the end of year report for this project submitted last year. Phosphotyrosine was measured as the most proximal readout for EGF-R activation. It was found that the addition of EGF led to an expected increase in phosphotyrosine (PY) for S1, S11 and 468. Unexpectedly the PY level never decreased in the 468 cell line but was rapidly decreased in the S1 or S11 variant cell lines. This result suggested that indeed, the negative regulatory apparatus was unable to regulate the amplified receptor number. The rate at which the EGF-R was down-regulated in these experiments correlated closely with the rate of PY decrease. This suggested that downregulation was one of the most important if not the most important negative regulatory pathway for attenuating activated EGF-R in these cells. However, the PY/EGFR ELISA results showed that in the S1 and S11 cell lines, PY was removed at a rate faster than EGF-R degradation occurred. For this reason, the effect of protein kinase C (PKC) and tyrosine phosphatases were also examined. Tyrosine phosphatases, but not PKC, appeared to be critical EGF-R negative regulatory components. The HMEC cell line A1 has an EGF-R per cell number (2×10^5) intermediate to that of the S variants and 468 cells but is not transformed. Thus, the A1 cells should be able to efficiently negatively regulate their entire EGF-R complement. Indeed, experimental results confirmed that this is the case. As was expected from the different EGF-R levels, the rate at which the A1 cells could eliminate activated receptors was faster than the 468 parental cell line but slower than either the S1 or S11 variants. The effect of phosphorylation at S1046/1047 was not determined because the most significant parts of the negative regulatory apparatus appeared to be EGF-R down-regulation and the activity of tyrosine phosphatases.

The primary reason for using both the S1 and S11 variants is to control for clonal differences. If both S1 and S11 behaved in a manner distinct from the parental 468 cell line but similar to each other, this would support the possibility that the observed differences were due to the different EGF-R levels and not to other differences. Because the karyotypes are not identical among the different cell lines, the EGF-R was amplified by retroviral mediated transduction in both the S1 and S11 variants. A bi-cistronic amphotrophic mouse retrovirus was used in these studies.

The EGF-R is inserted 5' of a G418 resistance cassette and the two genes are separated by an internal ribosome entry site (IRES). With this vector, barring a very rare recombination event, resistance to G418 will not occur in the absence of EGF-R expression.

Cells containing amplified EGF-R (designated XR for extra receptors) were cloned by limiting dilution and initially screened for induction levels by the binding of radioactive EGF or anti-EGF-R antibodies. Cell lines that had at least 3 fold higher EGF-R levels than either the S1 or S11 cell lines further screened by mini-Scatchard analysis in order to better determine the actual EGF-R amplification level. I125 labeled EGF was added to the amplified cell lines at a concentration of 0.98-250 ng/ml and allowed to come to equilibrium binding on ice. Cells were then washed with ice cold WHIPS buffer (pH 7.4) and the surface radioactivity was stripped off with a glycine/urea strip (pH 3.0). The number of counts stripped off the cells was determined by gamma counting and, after normalizing for cell number, used in Scatchard analysis (figure 1). The X intercept for each cell line corresponds to the number of receptors per cell and, because this is not a full Scatchard, are normalized to the X intercepts of S1 or S11. The clones S1XR13 (transduced clone 13 from variant S1 parental) and S11XR6 (transduced clone 6 from variant S11 parental) are both amplified approximately 8 fold over the S1 and S11 variants respectively. This level of amplification results in a final receptor per cell count of 1.28×10^5 for S1XR13 5.28×10^5 for S11XR6. These levels approximate the EGF-R level per cell of the A1 cells. Since these were the highest levels of amplification seen in any of the clones obtained, they were used in the remaining experiments.

If EGF-R amplification in the 468 parental cell line is responsible for its inability to efficiently downregulate activated receptors, then amplification of the EGF-R in the S1 or S11 cell lines should also decrease their ability to negatively regulate the EGF-R. Figure 2 compares the rate of decline in PY signal in the S1 cell line to that of S1XR13. Figure 3 compares the S11 cell line to that of S11XR6. All four cell lines were incubated with 100 ng/ml EGF for 0-360 minutes at 37 C. At each timepoint, the cells were lysed and EGF-R were immunoprecipitated. Proteins were resolved by SDS PAGE and probed using anti-PY antibodies. After quantification by

phosphoimage analysis, absolute PY levels were normalized to 100% at 10 minutes post EGF treatment. Figure 2 shows that the PY levels for S1XR13 decrease much more slowly than the PY levels for S1. Figure 3 shows that the PY levels for S11XR6 decline much more slowly than the PY levels corresponding to S11. These results recapitulate the results seen when S1 and S11 variants were compared to the 468 parental cell line, as expected. For this reason we conclude that amplification of the EGF-R, by itself, decreases the ability of the cell to negatively regulate receptor activity and may result in prolonged signaling.

In order to determine if the results seen in figures 2 and 3 were due to an inability to downregulate the EGF-R, the blots were stripped and reprobed with another anti-EGFR antibody. EGF-R levels were quantitated by phosphoimaging and then normalized to 100% at the 10 minute timepoint. Figure 4 compares the EGF-R levels in S1 to those of S1XR13. Figure 5 compares the EGF-R levels of S11 to those of S11XR6. As in the PY experiments, these results recapitulate the results seen when S1 and S11 were compared to the 468 parental cell line. The ability to attenuate active EGF-R corresponds most closely with the ability to downregulate EGF-R.

Since EGF-R amplification results in an increase in the number of activated EGF-R over time, we questioned whether or not these activated receptors were localized to signal. Several studies have shown that, at least in the case of the canonical Ras pathway, membrane localization is the only requirement for activation (19 and 20). It is thought that activation of EGF-R at the membrane allows it to signal through Ras and other pathways until it is internalized and removed from the signaling milieu (19 and 20). However, other researchers have suggested that endocytosed EGF-R also signal (17 and 18).

Figure 6 shows that the EGF-R on the surface of the 468 cell line are not efficiently internalized while the surface receptors on the S1 and S11 cells are efficiently internalized. Cells were treated with 100 ng/ml EGF for 0-120 minutes at 37 C. After incubation, the cells were placed on ice, washed with WHIPS and allowed to bind an I125 labeled non-antagonistic anti-EGF-R antibody for several hours. The antibody which binds cell surface EGF-R was then stripped off using a glycine/urea stripping solution. The number of counts stripped off the cell

surface were determined by gamma counting and normalized to cell number. Maximum surface counts (100%) occurs when no EGF has been added to the cells. After the addition of EGF, progressively larger numbers of EGF-R are induced to enter the cell and thus can no longer bind exogenously added labeled antibody. The absolute number of surface counts/cell (data not shown) suggests that most of the total receptor numbers for S1, S11 and 468 cells reside on the cell surface. In any case, the surface counts per cell for the 468 cells is always approximately 25-30 times that of S1 or S11. This shows that even if all receptors are not on the surface, 468 cells still have 30 fold more receptors on the cell surface than either variant.

Figure 6 shows that for S1 and S11, 70% of the surface receptors are internalized within 10 minutes following EGF treatment. 468 cells however, have only removed 33% of its total number of surface receptors after 10 minutes. After 120 minutes, 80% of both S1 and S11 surface EGF-R have been internalized. In contrast, after 120 minutes only 50% of the 468 surface receptors have been internalized. If all of the cells receptors are on the cell surface before EGF is added, then after a 120 minute treatment with EGF, the 468 cells still have 1×10^6 receptors on their surface. This is more receptors than either S1 or S11 started with and all of them are now active. Thus, the active 468 EGF-R are not efficiently removed from the cell surface following activation and are localized for signaling through canonical pathways.

Conclusions

- 1) The MDA-MB-468 variant cell lines S1 and S11 were transduced with EGF-R to a level of 1.28×10^5 receptors per cell for S1XR13 and 5.28×10^5 receptors per cell for S11XR6.
- 2) PY associated with activated EGF-R declines more slowly in the amplified variants (S1XR13 or S11XR6) than in either parent cell line (S1 and S11). This suggests that EGF-R amplification decreases the ability of the cell to negatively regulate its EGF-R.
- 3) The speed at which the cell can downregulate its EGF-R correlates with the cells ability to negatively regulate activated receptors. The rate at which S1XR13 and S11XR6 downregulate their EGF-R is much slower than the rate of S1 or S11 EGF-R downregulation.
- 4) Activated EGF-R are localized to signal through canonical signal transduction pathways. Activated EGF-R are removed from the cell surface rapidly in S1 or S11 cells but very slowly in the MDA-MB-468 cell line.

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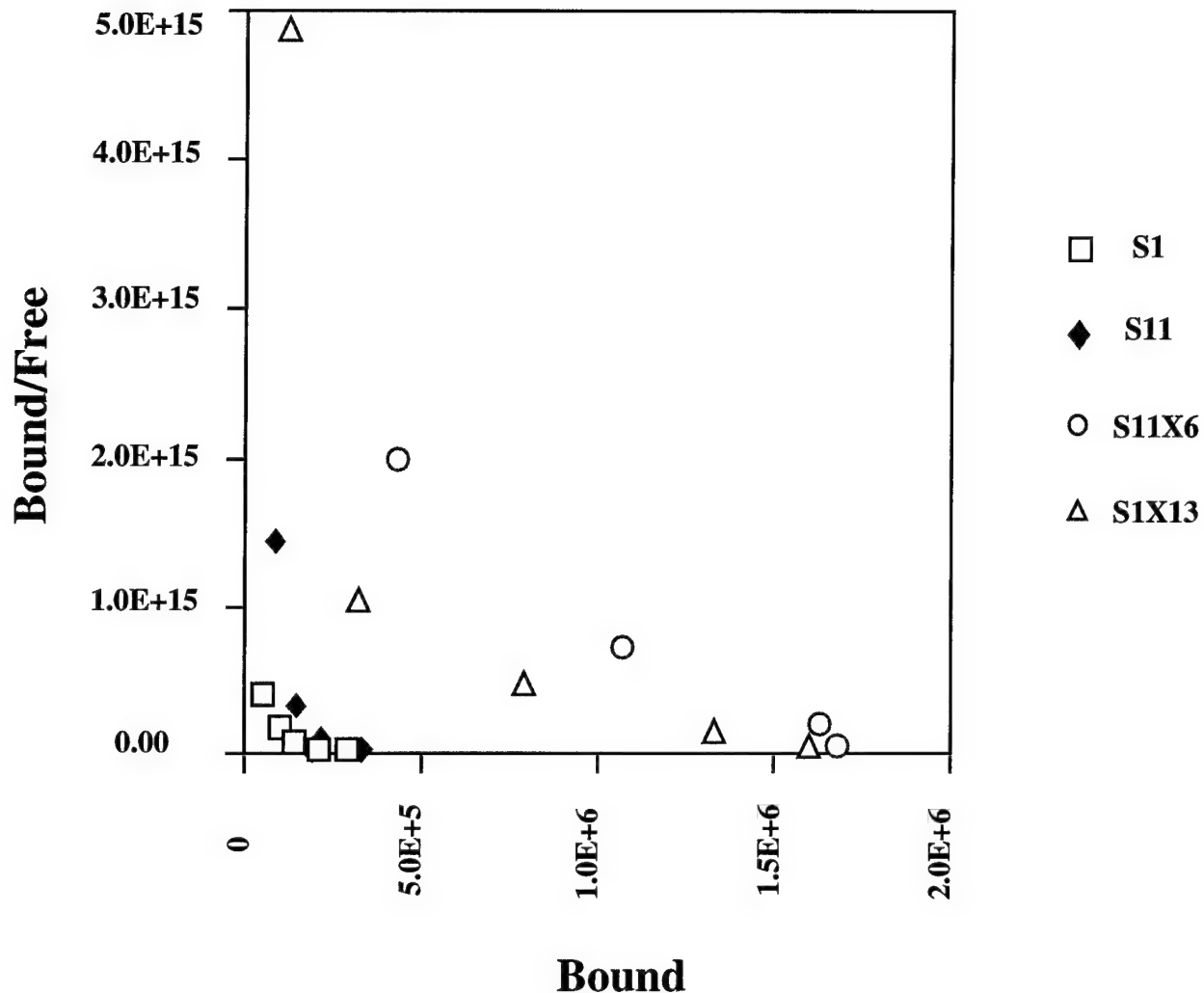


Figure 1: Mini-Scatchard analysis of EGF-R transduced variant cell lines. EGF-R transduced S1 and S11 cell lines were placed on ice and washed 3 times each with ice cold WHIPS pH 7.4 (0.1% Polyvinylpyrrolidone, 130 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂-6H₂O, 1 mM CaCl₂-2H₂O, 20 mM HEPES (Free acid)). 1 ml I125 labeled EGF diluted serially: 250 ng/ml, 62.5 ng/ml, 15.6 ng/ml, 3.9 ng/ml, and 0.98 ng/ml in DVHB (DV media supplemented with 0.1% BSA) was added to each plate and incubated for 7.5 hours on ice. After incubation, the plates were again washed 4 times with ice cold WHIPS. 1 ml ice cold glycine/urea strip pH 3.0 (50 mM glycine, 100 mM NaCl, 1 mg/ml Polyvinylpyrrolidone, 2 M Urea) was added to each plate incubated for 5 minutes and then collected into a gamma tube. Plates were then rinsed once with 1 ml of glycine/urea strip pH 3.0 and the wash was also collected into the gamma tube and read in a gamma counter. Cell number was determined using a Coulter counter.

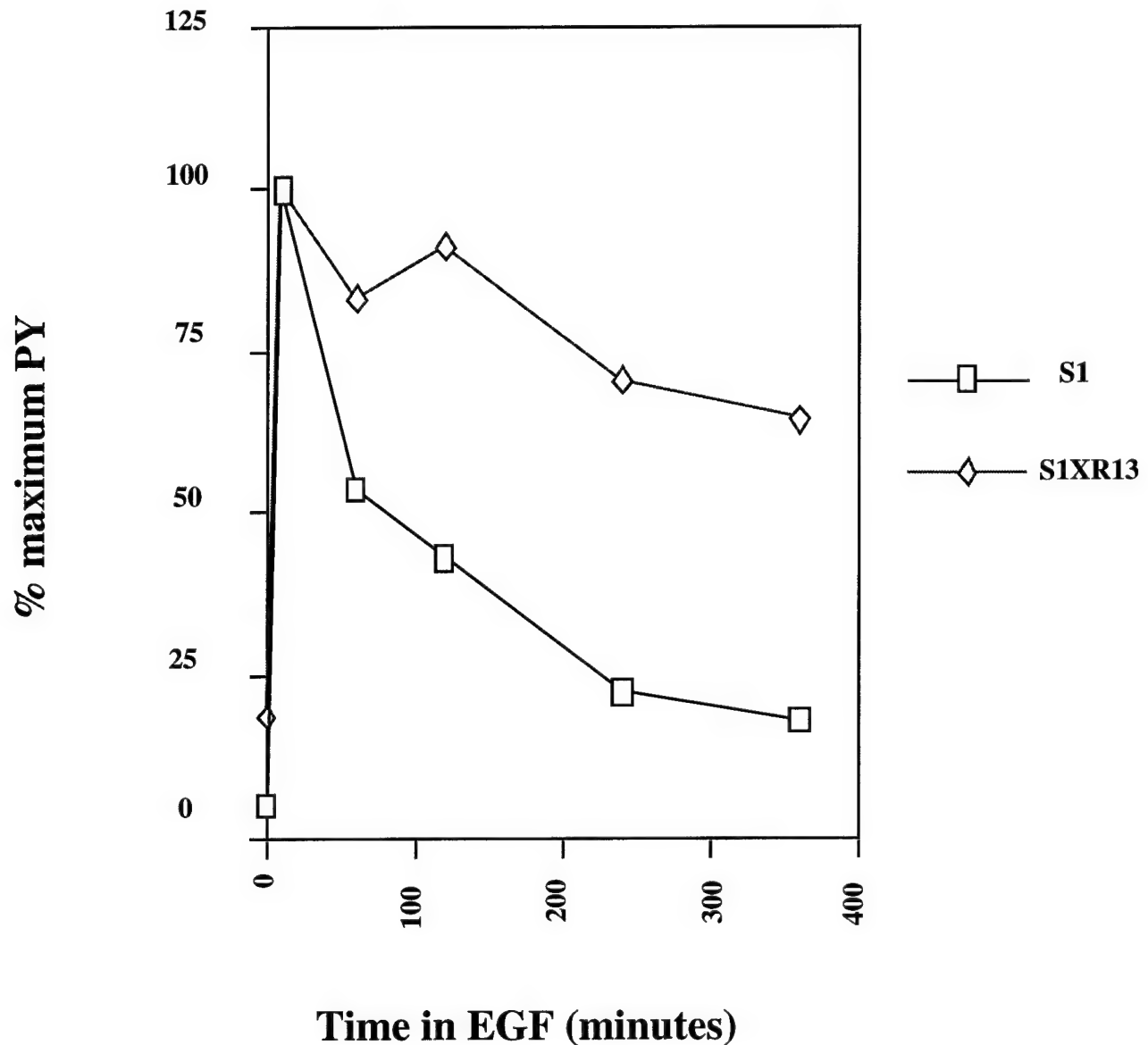


Figure 2: Activated receptors are attenuated faster in S1 than in its amplified variant S1XR13. S1XR13 and S1 cells were treated with 100 ng/ml rHEGF for 0-360 minutes at 37 C. EGF-Rs were extracted in RIPA (150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% Sodium dodecyl sulfate, 50 mM Tris pH 7.2, 4 mM Iodoacetate, 1 mM Orthovanadate, 10 ug/ml Pepstatin, Chymostatin, Leupeptin and Aprotinin), immunoprecipitated with anti-EGF-R antibodies (225), and resolved by SDS PAGE. Phosphotyrosine was visualized using RC20 anti-phosphotyrosine antibody and chemiluminescent detection.

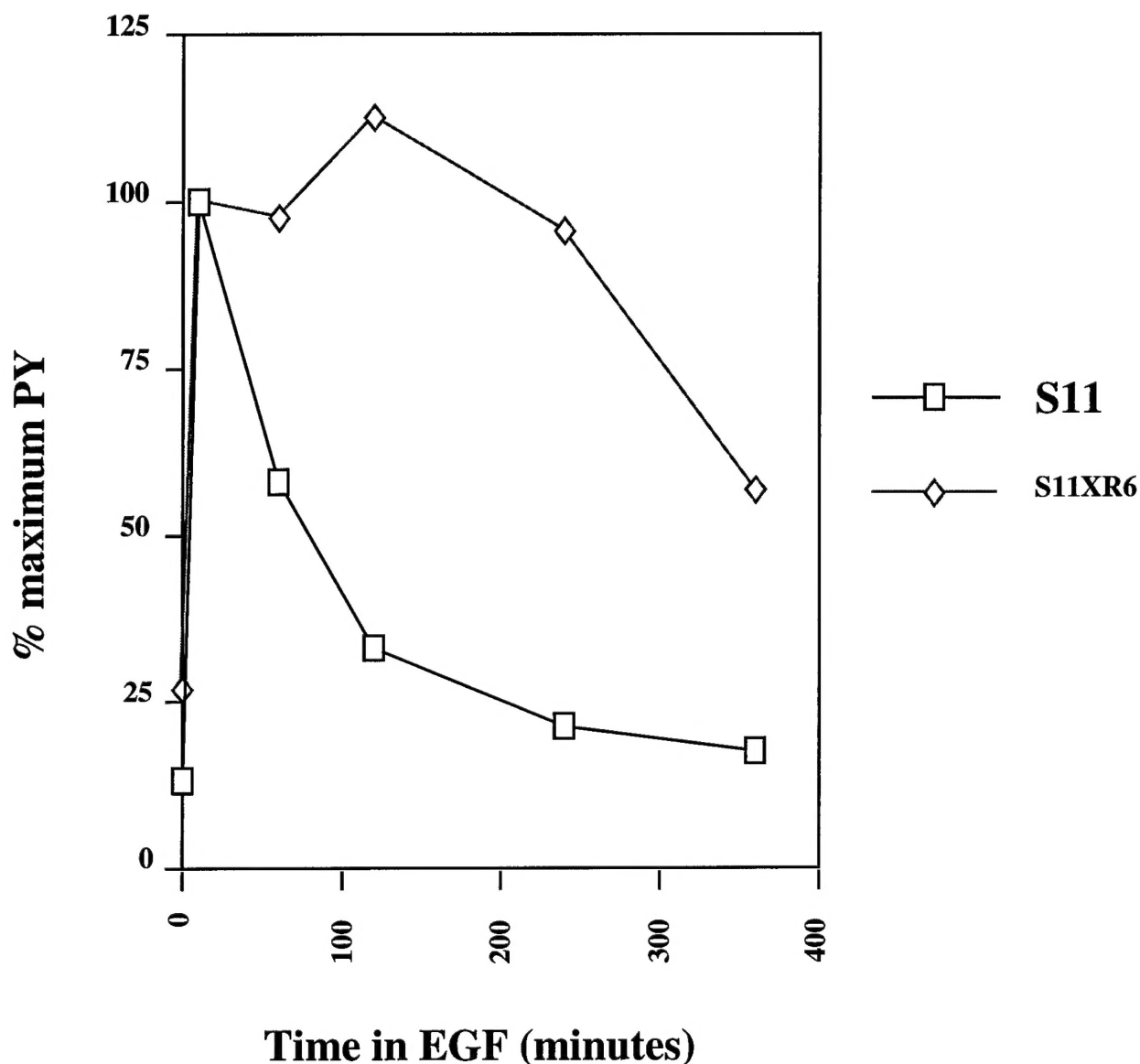


Figure 3: Activated receptors are attenuated faster in S11 than in its amplified variant S11XR6. S11XR6 and S11 cells were treated with 100 ng/ml rHEGF for 0-360 minutes at 37 C. EGF-Rs were extracted in RIPA (150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% Sodium dodecyl sulfate, 50 mM Tris pH 7.2, 4 mM Iodoacetate, 1 mM Orthovanadate, 10 ug/ml Pepstatin, Chymostatin, Leupeptin and Aprotinin), immunoprecipitated with anti-EGF-R antibodies (225), and resolved by SDS PAGE. Phosphotyrosine was visualized using RC20 anti-phosphotyrosine antibody and chemiluminescent detection.

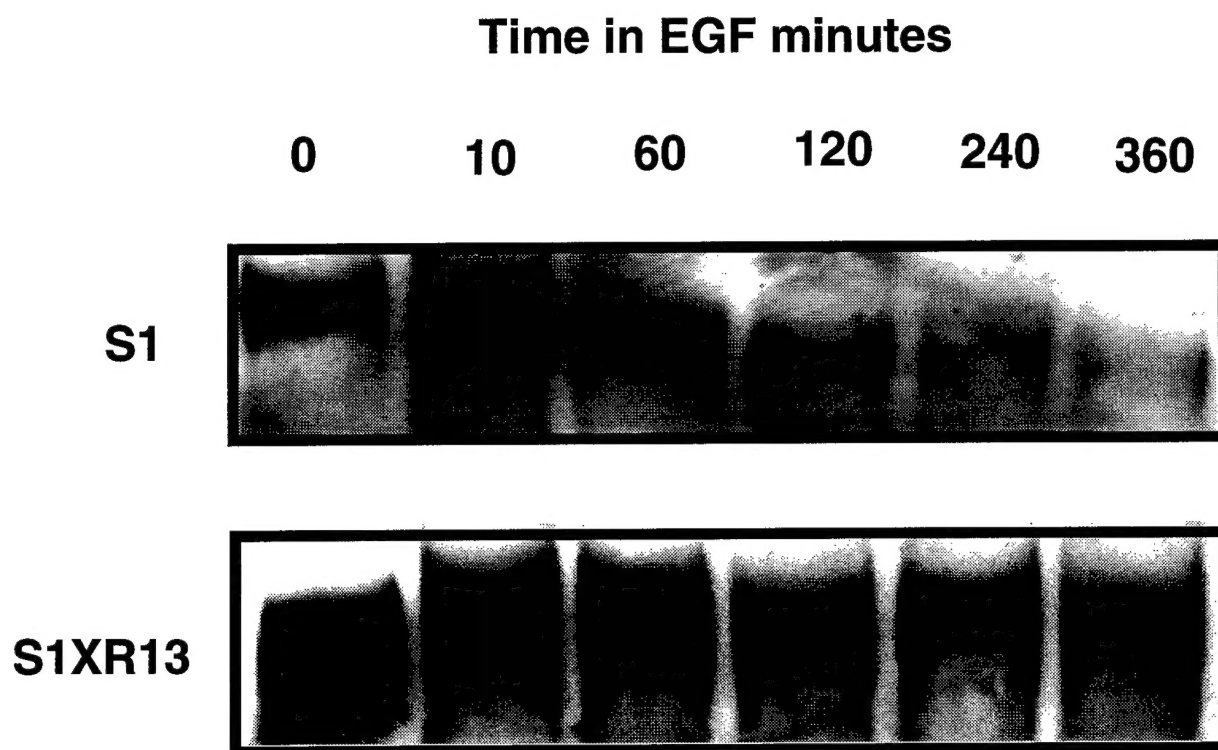


Figure 4: The EGF-R transduced cell line S1XR13 downregulates the receptor much more slowly than the parental cell line S1. The anti-Phosphotyrosine blot shown in figure 2 was stripped (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris HCl pH 6.7) for 30 minutes at 55 C, and reprobbed with a different anti-EGF-R antibody distinct from the immunoprecipitation antibody (N13). The receptor antibody was visualized with an anti-Rabbit HRP conjugated antibody detected using chemiluminescence.

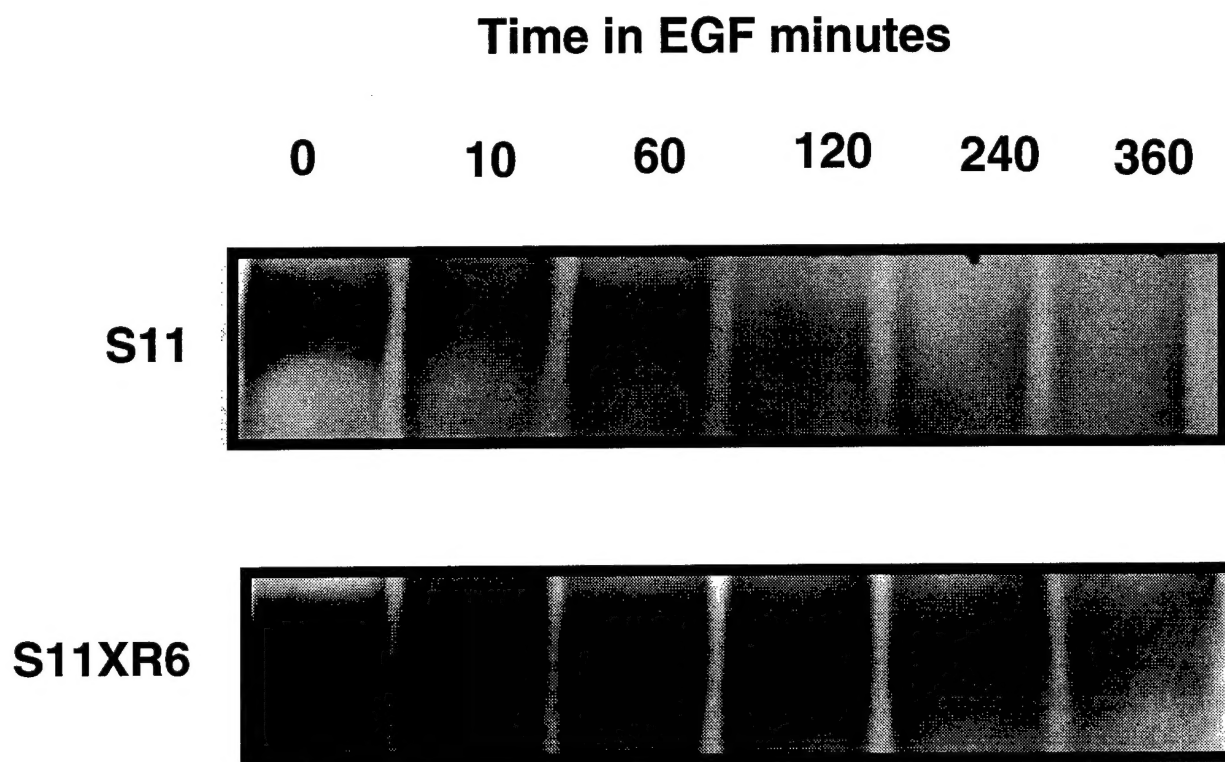


Figure 5: The EGF-R transduced cell line S11XR6 downregulates the receptor much more slowly than the parental cell line S11. The anti-Phosphotyrosine blot shown in figure 3 was stripped (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris HCl pH 6.7) for 30 minutes at 55 C, and reprobed with a different anti-EGF-R antibody distinct from the immunoprecipitation antibody (N13). The receptor antibody was visualized with an anti-Rabbit HRP conjugated antibody detected using chemiluminescence.

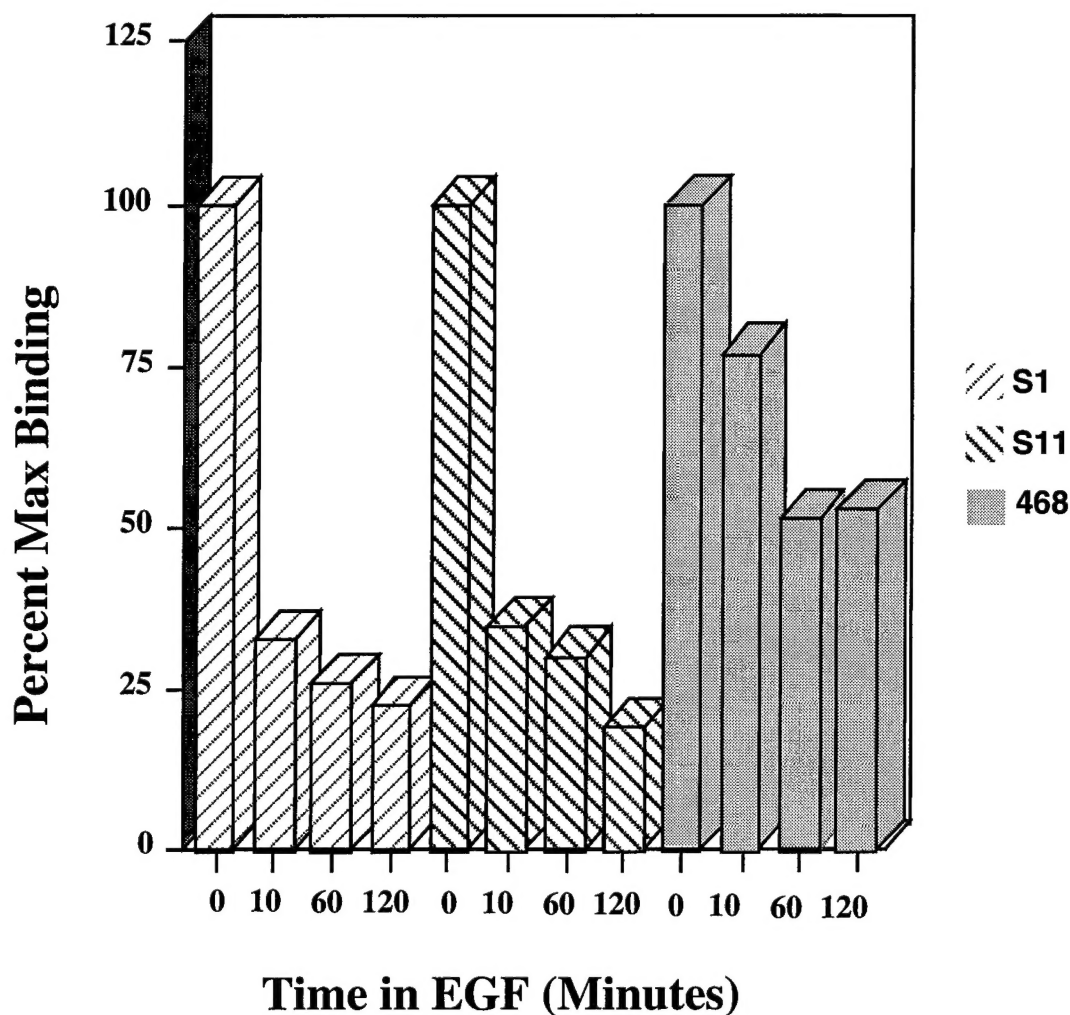


Figure 6: Activated EGF-R is rapidly removed from the surface of S1 and S11 variants but not from the surface of the parental 468 cell line. 100 ng/ml EGF was added to S1, S11 or 468 cell media for 0-120 minutes at 37C. After incubation, the cells were placed on ice and washed 3X with ice cold WHIPS pH 7.4 (0.1% Polyvinylpyrrolidone, 130 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂-6H₂O, 1 mM CaCl₂-2H₂O, 20 mM HEPES (Free acid)). 1 ml of 3 ug/ml I125 labeled 13A9 was added to each plate. Plates were then incubated for 2 hours at 4 C. After incubation, plates were again washed 3 times with ice cold WHIPS. 1 ml ice cold glycine/urea strip pH 3.0 (50 mM glycine, 100 mM NaCl, 1 mg/ml Polyvinylpyrrolidone, 2 M Urea) was added to each plate and allowed to incubate for 5 minutes and then collected into a gamma tube. Plates were then rinsed once more with another 1 ml of glycine/urea strip and this wash was also collected into the gamma tube. All samples were counted in a gamma counter. Cell number was determined by counting in a Coulter counter.